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Authors
Dell, Karen R
Vale, Ronald D

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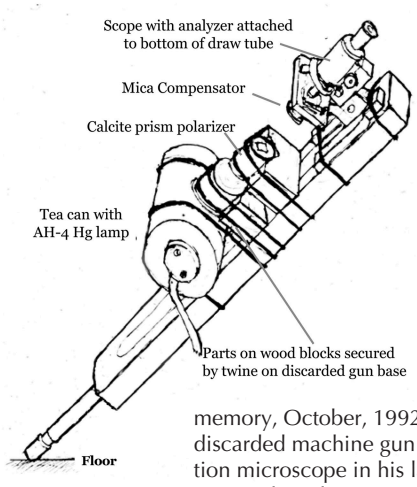
A tribute to Shinya Inoue and innovation in light microscopy

Karen R. Dell[1] and Ronald D. Vale[2]

[1]Reviews Editor, The Journal of Cell Biology
[2]Department of Cellular and Molecular Pharmacology and the Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA

The 2003 International Prize for Biology was awarded to Shinya Inoue for his pioneering work in visualizing dynamic processes within living cells using the light microscope. He and his scientific descendants are now pushing light microscopy even further by developing new techniques such as imaging single molecules, visualizing processes in living animals, and correlating results from light and electron microscopy.

In 1943, under the darkness of air raid black-out curtains in Tokyo, the great cell biologist Katsuma Dan posed a challenge to his young student Shinya Inoue: develop a microscope to visualize the birefringence of the mitotic spindle in sea urchin eggs. Spindle birefringence, the phenomenon of light polarization by parallel spindle fibers, was first reported by W. J. Schmidt in 1939 (Schmidt, 1939). But developing and improving such methodology in the setting of war-torn Japan constituted a challenge of considerable magnitude. Nevertheless, Inoue succeeded in constructing his first polarizing microscope in 1947, using a discarded machine gun base to align the optics and a tin can as housing for the light source (Fig. 1). This microscope, nicknamed the Shinya Scope, marked the beginning of a 60-yr career of innovation in microscopy that is still ongoing at the Marine Biological Laboratories at Woods Hole, MA. Inoue and his current microscope are shown in Fig. 1.



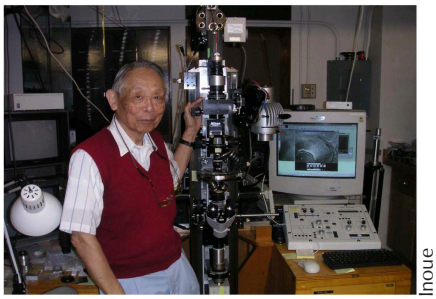

Inoue

Figure 1. **Shinya Inoue and his microscopes.** (Left) A diagram of Inoue's polarization microscope from 1947 (reconstructed from memory, October, 1992). The optical components were mounted on a discarded machine gun barrel. (Right) Inoue and his current polarization microscope in his laboratory at the Marine Biological Laboratory at Woods Hole, MA.

Inoue's contributions to microscopy and cell biology were recognized by his receipt of the prestigious International Prize for Biology in 2003. The Japan Society for the Promotion of Science (JSPS) awards this prize annually to "an individual who, in the judgment of the members of the Committee, has made an outstanding contribution to the advancement of research in fundamental biology." In the 19-yr history of the prize, this is only the second time that the prize has been awarded for work in cell biology. To pay tribute to Inoue and to celebrate this honor, an international conference entitled Cellular and Molecular Dynamics Revealed by Advanced Light Microscopy was organized by Toshio Yanagida (Osaka University, Osaka, Japan) and held in Nara, Japan, in December, 2003. Here, we will highlight some of Inoue's achievements that led to his selection for the International Prize and also highlight a subset of the 25 presentations made at this meeting.

The great microscopists of the 19th and 20th century (e.g., Edmund B. Wilson, Walther Flemming, and Theodor Boveri) produced captivating images of cells and subcellular organelles such as the centrosome and mitotic spindle (Harris, 1999). However, these images were generally produced in fixed cells that were impregnated with various stains. Inoue's mission and great contribution was to pioneer the imaging of live cells, realizing that the dynamics of cellular processes must be recorded in order to understand mechanism. Students who have grown up with GFP imaging may take this idea for granted, but in the 1940s and 1950s this approach of live cell imaging was revolutionary and largely uncharted. Moreover, new techniques had to be developed to image the fine details of subcellular structures in liv-

Correspondence to kdell@itsa.ucsf.edu

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ing cells. For cell division and mitosis, Inoue's long-term interest, the machinery responsible for the movement of chromosomes in mitosis/meiosis could not be detected in living cells with the standard bright-field or phase-contrast imaging used at the time. For example, the "fibers" (now known to be bundles of microtubules) of the mitotic spindle could only be seen with certain procedures of fixation/staining and could not be visualized in living cells. Inoue's home-built polarizing microscopes, however, succeeded in visualizing the mitotic spindle fibers in a variety of healthy cells and demonstrated that these fibers are indeed responsible for the motions of chromosomes (Inoue, 1953).

Dynamic observations of the mitotic spindle fibers made by Inoue and his students had a major impact on mitosis research and remain germane to the thinking of the field to this day. In the mid-1960s, Inoue and colleagues, with his innovation of rectified optics to correct for the depolarization of light by the objective lens, obtained improved polarization images. Aided by these improvements, they showed that the fibers and their constituent fibrils (later proven to be microtubules) in the mitotic spindle could reversibly polymerize and depolymerize and that this equilibrium could be shifted toward depolymerization by treatments such as cold temperature and colchicine, or toward polymerization by D_2O (Inoue and Sato, 1967). Indeed, these observations stimulated Ed Taylor and his students to use radioactive colchicine to identify tubulin as the protein that composed the spindle (Weisenberg et al., 1968). After the purification of tubulin, Inoue's observations of polymerization/depolymerization of the mitotic spindle fibers could be replicated with pure tubulin in a test tube (Weisenberg, 1972; Olmsted and Borisy, 1975). These studies represented perhaps the first case where the dynamics of a subcellular structure (the microtubule) was dissected both in vivo and in vitro, a paradigm that still poses a contemporary challenge for biologists. Inoue and colleagues also observed that the chromosomes were displaced by the shortening and lengthening of the fibers when the various perturbations described above were applied. This observation led Inoue to speculate that polymerization/depolymerization reactions generated the forces that push or pull chromosomes during prometaphase and anaphase. This idea was met initially by much skepticism, but it fueled a great deal of research and is increasingly gaining acceptance in the mitosis field (Rogers et al., 2004).

Another remarkable achievement by Inoue (and also independently by Robert Allen) was the development of video microscopy, the use of a video camera, instead of the eye, to record images from the microscope (Inoue, 1981). Inoue demonstrated that video, combined with computer-assisted contrast enhancement, provided a new clarity and visualization of detail within cells that was never before achieved. Of course, this method spread like wild fire through the scientific community and has relegated the eye piece in most modern microscopes to a focusing device. Inoue also authored two editions of a book entitled *Video Microscopy*, which have been translated into Spanish and Japanese and have become the definitive work on this methodology for the past two decades (Inoue and Spring, 1997).

In the symposium honoring Inoue's award and his pioneering spirit in light microscopy, new developments in microscopy were presented encompassing topics from the dynamics of single molecules to the behavior of cells in the tissues of living animals. Unable to discuss all of the talks, we highlight a subset of the presentations here.

Single molecule analysis of cell signaling

One of the great advances in light microscopy has been the ability to observe the behavior of individual molecules using various microscopy techniques, such as optical trapping and total internal reflection fluorescence (TIRF) microscopy. Indeed, such techniques have been highly refined to measure forces, individual steps, and conformational changes of motor proteins in vitro as highlighted in the talks by Yale Goldman (University of Pennsylvania, Philadelphia, PA), Toshio Yanagida (Osaka University), and Kazuhiko Kinosita (Okazaki National Research Institute, Okazaki, Japan). Adding to the armament of methods, Toshio Ando (Kanazawa University, Kanazawa, Japan) presented his laboratory's efforts to perform real time "imaging" of single molecules in vitro with a high-speed atomic force microscope and highlighted their recent success in using this technique to follow the motion of single kinesin molecules along a microtubule (Ando et al., 2003).

The new frontier of imaging single molecules within living cells and the use of such methods for understanding signaling pathways was also featured. Several investigators applied single molecule methodology to the epidermal growth factor (EGF) signaling pathway, but approached the problem in different ways. Yasushi Sako and Toshio Yanagida (Osaka University) imaged Cy3-labeled EGF by single molecule TIRF microscopy and could visualize individual binding events of the ligand to its receptor. These investigators provided evidence for receptor dimerization events by documenting an approximately bimodal fluorescence intensity distribution of Cy3-EGF bound to the membrane as well as by showing colocalization and FRET between Cy3-EGF and Cy5-EGF (Sako et al., 2000). In recently published work (Hibino et al., 2003), these investigators examined the spatiotemporal dynamics of Raf-GFP, a downstream signaling protein in the pathway, and showed that EGF receptor activation decreases the rate of dissociation of Raf-GFP from specific sites on the plasma membrane where prominent morphological changes occur. Thus, this downstream activator is recruited, in a spatially regulated manner, through a shift in its equilibrium binding constant for plasma membrane components.

Elizabeth Jares-Erijman (University of Buenos Aires, Buenos Aires, Argentina) and Thomas Jovin (Max Planck Institute, Göttingen, Germany) presented another approach for imaging

single EGF molecules on the cell surface by labeling the ligand with a quantum dot. Quantum dots (QDs) are nanometer-sized, semiconductor particles that emit bright fluorescence with little photobleaching, and QDs derivatized with, for example, streptavidin are now commercially available. Jares-Erijman, Jovin, and their colleagues conjugated biotinylated-EGF to streptavidin-QD particles and could follow individual EGF-QD particles by confocal microscopy (Lidke et al., 2004). One of their interesting findings was that EGF receptors connected to the actin cytoskeleton in a process that required receptor signaling, and the receptors exhibited retrograde flow from the tips to the base of filopodia.

Concentration of receptors and effector proteins into plasma membrane microdomains called lipid rafts has been suggested to be an important part of signaling cascades in many systems. However, in resting cells, lipid rafts are thought to be small and to form transiently and dissociate, making them difficult to study (Lai, 2003). Akihiro Kusumi (Nagoya University, Nagoya, Japan) and his colleagues presented their work on studying and defining lipid rafts using single molecule observation of a raft-associated protein called CD59, which they tracked with anti-CD59 Fab fragments linked to a nanometer-sized gold particle. Their studies with CD59, as well as other raft-associated proteins, show that proteins undergo transitions from relatively unconstrained two-dimensional diffusion to temporary immobilization in transient confinement zones (TCZs). These TCZs may represent lipid raft microdomains, which also are anchored to the actin cytoskeleton. Kusumi and colleagues then performed dual imaging of the anti-CD59 gold particles along with fluorescence imaging of GFP-tagged signaling proteins, their goal being to see if the TCZ event correlated with the recruitment of specific proteins. Indeed, they found that $G\alpha_{1,2}$-GFP colocalized with the anti-CD59 gold particle slightly before the onset of TCZ formation, suggesting perhaps a role in causing the TCZ. Certain proteins (e.g., PLCγ) colocalized at the onset of confinement, whereas others (Lyn-GFP) colocalized at later times. This work suggests that there may be an ordered set of temporal events involved in formation of lipid rafts and in the subsequent recruitment of molecules to the raft.

Dynamics of subcellular structures

Although single molecule imaging is powerful, sometimes imaging a "handful" of fluorescent molecules can be the method of choice. Clare Waterman-Storer (The Scripps Research Institute, La Jolla, CA) and Ted Salmon (University of North Carolina, Chapel Hill, NC) described how low expression levels of GFP-actin or GFP-tubulin result in stochastic incorporation in polymers, thereby giving rise to an uneven or "speckled" fluorescence appearance of the polymers. These speckles provide fiduciary markers that can be followed over time, allowing one to visualize "treadmilling" of subunits within actin or microtubule polymers or to detect physical motion of the polymers (Waterman-Storer et al., 1998). Waterman-Storer discussed new algorithms, developed in collaboration with Gaudenz Danuser (The Scripps Research Institute), to analyze fluorescent speckle microscopy movies (Ponti et al., 2003). These algorithms allow the creation of spatial maps of actin assembly and disassembly, in localized regions of a contact inhibited cell, as well as in cells undergoing morphogenesis and migration. Salmon described how speckle microscopy can be used to study the dynamics of forces acting on the kinetochore, a question that he has pursued since he was a graduate student with Inoue. Salmon showed that, in metaphase, kinetochore-attached microtubules are constantly polymerizing at the same rate as poleward flux, thereby producing tension—by resistance to the flux—on the kinetochore attachment site (Maddox et al., 2003). During anaphase when the chromosomes separate, the drop in tension results in microtubule depolymerization at the kinetochore, which, along with poleward flux, produces chromosome separation.

Roger Tsien (University of California, San Diego, CA) described how light microscopy can be used to distinguish "old" proteins from newly synthesized proteins, thus providing a powerful technique for understanding the turnover of subcellular compartments. This approach involves expressing a recombinant protein of interest with a tetracysteine tag that will bind covalently to a cell-permeable, biarsenical green (FlAsH) or red (ReAsH) fluorophore. First, all of the tagged proteins in the cell are saturated with an excess of one of the fluorophores (let us say the green FlAsH), then the unbound fluorophore is removed by washing. After an adjustable delay to allow for new protein synthesis, the other colored fluorophore (ReAsH) is added. The second fluorophore only binds to the newly synthesized protein that has an unoccupied tetracysteine motif, thereby providing a visual image of "old" (green in this example) and "new" (red) proteins. Previous work using this method established the mechanism of protein exchange in gap junctions (Gaietta et al., 2002). In newer work, involving a collaboration between Tsien and Rob Malenka (Stanford University, Stanford, CA), this technique was used to demonstrate that synthesis of AMPA receptors (key molecules implicated in learning and memory) can occur within the distal dendrites of a neuron even after isolation from the cell body.

Dynamics of cells in living animals

One of the exciting new directions for light microscopy is watching molecular dynamics in the tissues of living animals, and Winfried Denk (Max Planck Institute, Heidelberg) and Jeff Lichtman (Washington University, St. Louis) both provided tantalizing tastes of things to come. Denk showed how two-photon excitation provides optical sectioning capability to visualize neuronal activity, even relatively deep into brain or other neuronal tissues. Using calcium-sensing dyes, Denk and colleagues imaged spatiotemporal patterns of dendritic excitation of starburst amacrine cells in intact retinal preparations in response to light stim-

uli. Denk also showed two-photon fluorescence images of individual neurons ≥1 mm deep in the neocortex of a living mouse brain, a feat that was achieved by high-energy femtosecond pulse excitation (Theer et al., 2003). With the ability to make transgenic mice expressing calcium-sensing GFP proteins in specific neurons, this technology has great potential for understanding neural networks, since it allows large numbers of neurons in a functioning brain to be measured optically, rather than using more laborious electrophysiological methods.

On a similar theme, Lichtman and coworkers visualized the process of motor neuron innervation in muscle fibers in a live mouse expressing GFP-Thy1 (an abundant membrane protein). It is well known that individual muscle fibers of newborn animals are innervated by multiple neurons; however, in the first two weeks after birth, the redundant innervating synapses are pruned and finally each muscle maintains a synaptic connection with just one axon. By repeatedly imaging the same synapse over several days, Lichtman and colleagues were able, for the first time, to make a time-lapse movie of this synaptic elimination process (Walsh and Lichtman, 2003). These investigators visualized axonal branches extending or retracting on a rapid time scale. Moreover, when one axon withdrew from a site, a competing axon grew to colonize the empty niche. Although unpredictable at the beginning, one axon eventually emerges as the victor, forming more connections on the fiber and displacing its competitors.

Combining information from light and electron microscopy

An unexpected but exciting aspect of this "light microscopy" symposium was the resurgence of electron microscopy and its utility in conjunction with light microscopy. Gary Borisy (Northwestern University, Chicago, IL) demonstrated how the two methods provide complementary information on how actin networks reorganize into filopodia, dynamic cellular protrusions containing actin filament bundles as internal struts. Using live cell imaging with GFP-actin, Borisy and colleagues followed successive stages in the maturation of a filopodium (Svitkina et al., 2003). These investigators then fixed and prepared the same cells for electron microscopy, so that they could examine the actin organization associated with the birth, "adolescence," and maturation of a filopodium, as defined by their live cell imaging. Their results showed that actin filaments were clustered and in register at the tips of a nascent filopodium, but the bundling of actin filaments farther below the membrane occurred after a delay. This is consistent with their live cell imaging experiments showing that GFP-tagged vasodilator-stimulated-phosphoprotein (GFP-VASP) formed foci early in filopodia formation—suggesting that it is a key molecule in the filopodia "tip" complex—whereas GFP-fascin (an actin bundling protein) accumulated only later in the process.

Tsien described how ReAsH (described earlier) can be used for correlating light and electron microscopic images. Not only is ReAsH fluorescent, but under intense illumination in a fixed specimen, it can induce the polymerization of diaminobenzidine (DAB) into an insoluble precipitate that can be visualized by electron microscopy. Tsien and colleagues previously used this technology to study the trafficking of connexin (the gap junction channel) to the cell surface at the light and EM level (Gaietta et al., 2002). At this meeting, Tsien presented new work showing this methodology could be applied to address the controversial question of what happens to the Golgi apparatus in mitosis; some investigators have proposed that it fuses with the ER, whereas others have maintained that the Golgi fragments and the pieces retain a separate identity from the ER. Using a tetracysteine-tagged Golgi protein labeled with ReAsH, these workers imaged Golgi fragmentation during mitosis by light microscopy and used ReAsH-induced photoconversion of DAB to show by EM that Golgi fragments remained almost completely separate from the ER in mitotic cells.

Summary

Inoue's life work has illustrated the success of developing new technologies in microscopy to answer critical questions in cell biology. Such a strategy is now being adopted by many investigators, and the explosion of new information on cellular dynamics was illustrated by the talks at this symposium in Inoue's honor. However, there is much more to come. As Inoue said in his talk, "Light microscopy has uncovered some surprising behaviors of dynamic structural molecules in living cells. Still, we have only scratched the surface. We look forward to growing interdisciplinary innovations in microscopy based on keener insights into the interaction of polarized light with matter that certainly should reveal ever finer mechanisms underlying the mysteries of nature, and of life itself." At 83-yr young, Inoue, with his love of science, his continued keen insight into cell biology, and his unwavering support of young scientists, continues to teach us a great deal about biology, microscopy, and how to conduct ourselves in the pursuit of scientific discovery. The 2003 International Prize for Biology could not have been bestowed on a nobler scientist and individual.

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